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Chemical Genetics Reveals a Role of Squalene Synthase in TGFβ Signaling and Cardiomyogenesis

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Abstract: *KY02111 is a widely used small molecule that boosts cardiomyogenesis of the mesoderm cells derived from pluripotent stem cells, yet its molecular mechanism of action remains elusive. The present study resolves the initially perplexing effects of KY02111 on Wnt signaling and subsequently identifies squalene synthase (SQS) as a molecular target of KY02111 and its optimized version, KY-I. By disrupting the interaction of SQS with cardiac ER-membrane protein TMEM43, KY02111 impairs TGFβ signaling, but not Wnt signaling, and thereby recapitulates the clinical mutation of TMEM43 that causes arrhythmogenic right ventricular cardiomyopathy (ARVC), an inherited heart disease that involves a substitution of myocardium with fatty tissue. These findings reveal a heretofore undescribed role of SQS in TGFβ signaling and cardiomyogenesis. KY02111 may find its use in ARVC modeling as well as serve as a chemical tool for studying TGFβ/SMAD signaling.*

Introduction

The heart is the first organ to become functional in the vertebrate embryo.[1] Although the heart develops early, cardiogenesis is a highly regulated process involving differentiation and cellular specialization. Cardiomyocytes comprise approximately 40 % of the total heart cells and originate from mesoderm cells through an organized interplay of extracellular factors including members of the bone morphogenetic proteins (BMPs), including Activin and NODAL, fibroblast growth factor (FGF), and Wingless (Wnt) families.[1,2] Our understanding of the cardiomyogenesis complexity has greatly been enhanced by in vitro modeling using pluripotent stem cells (PSCs); however, challenges remain regarding efficiency of differentiation and maturation for therapeutic applications and disease modeling.

Our previous screening of a chemical library and structure optimization led to the discovery of KY02111 (Figure 1 A), a small-molecule probe that boosts cardiomyogenesis of a range of PSCs including humans.[3] Brief 3-day exposure of PSC-derived mesoderm cells to KY02111 elicits the differentiation to cardiomyocytes. Despite the utility of KY02111 in

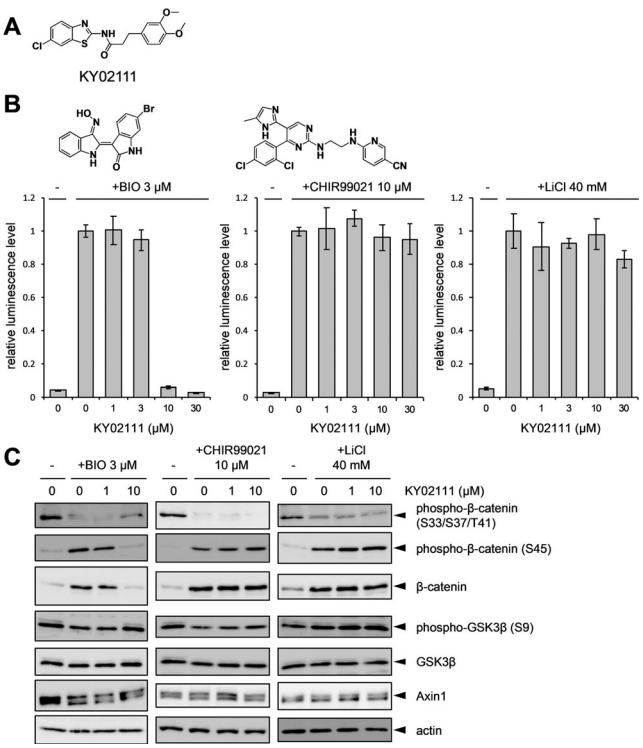

Figure 1. Analysis of KY02111's effects on Wnt signaling. A) Structure of KY02111. B) Effects of KY02111 on GSK3β inhibitor-induced TCF promoter activation. HEK293 cells were transfected with TOPflash and β-galactosidase plasmids and cultured for 24 h. The cells were then treated with various concentrations of KY02111 in a medium containing BIO (3 μM), CHIR99021 (10 μM), or LiCl (40 mM). After 24 h, luciferase activity was measured. Values are mean ± SD. ($n = 3$) C) Effects of KY02111 on the Wnt signal proteins. HEK293 cells were treated with various concentrations of KY02111 in a medium containing BIO (3 μM), CHIR99021 (10 μM), or LiCl (40 mM) for 24 h. The lysates were immunoblotted with the indicated antibodies. The samples were derived from the same experiment, and they were analyzed in parallel.

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the xeno-free preparation of cardiomyocytes, the mechanistic underpinning for its effect on the cardiomyogenesis remains incompletely understood. Our previous study showed that KY02111 potently blocked the Wnt signaling that is pharmacologically induced by BIO, a small-molecule GSK3β inhibitor. The Wnt signaling inhibition of KY02111 appears consistent with the earlier notion that, although Wnt signaling is essential during the initial stage of PSC differentiation to mesoderm cells, its repression increases the yield of cardiomyocytes at later stages.[4] However, since our earlier transcriptome analysis showed a marginal effect of KY02111 in the downregulation of the Wnt-dependent genes, its effects on the Wnt target genes could not be clearly established.[3]

Here we resolve the initially perplexing effects of KY02111 on Wnt signaling and then identify squalene synthase (SQS) as a *bona fide* molecular target of KY02111. The present study demonstrates that KY02111 primarily impairs TGFβ signaling, but not Wnt signaling, by disrupting the interaction of SQS with cardiac ER-membrane protein TMEM43. Our study of KY02111 and their analogs highlights a heretofore unknown role for SQS in TGFβ signaling and cardiomyogenesis.

Results

KY02111 Does Not Inhibit Wnt Signaling

Canonical Wnt signaling is tightly regulated by the amount of the transcriptional co-activator β-catenin that dictates key developmental gene expression patterns. In the absence of Wnt, cytoplasmic β-catenin protein is constantly degraded by the action of the Axin complex, which contains casein kinase 1 (CK1) and glycogen synthase kinase 3β (GSK3β). CK1 and GSK3 sequentially phosphorylate the amino terminal region of β-catenin at Ser45 and Ser33/Ser37/Thr41, respectively, resulting in β-catenin recognition by β-TrCP, an E3 ubiquitin ligase subunit, and subsequent β-catenin degradation.[5,6] This continual elimination of β-catenin leads to the repression of Wnt target genes. When a Wnt ligand binds to a transmembrane Frizzled (Fz) receptor and its co-receptor, low-density lipoprotein receptor related proteins (LRPs), the Wnt-Fz-LRP membrane complex squelches the Axin complex to the membrane, leading to the stabilization of β-catenin and the subsequent activation of Wnt target genes.[7]

The Wnt signaling is readily impaired by two well-known chemical inhibitors: XAV939 (tankyrase inhibitor)[8] and IWP-2 (porcupine inhibitor).[9] These two potent inhibitors have been shown to promote the differentiation to cardiomyocytes from PSC-derived mesoderm cells.[10] Since their target proteins are located upstream of β-catenin in the Wnt signaling pathway, their cardiomyogenesis activity can be canceled by adding (2′Z,3′E)-6-bromoindirubin-3′-oxime (BIO), a Wnt signal activator that inhibits GSK3β.[11] In contrast, the addition of BIO has no impacts on the cardiomyogenesis activity of KY02111. This observation previously led us to believe that KY02111 acts on the downstream of the Wnt signaling pathway.[3] In fact, KY02111 potently inhibited the ability of BIO to drive a reporter gene in which the expression of luciferase is controlled by TCF, a far downstream transcription factor of the Wnt signaling.[3]

We examined the effects of KY02111 on the ability of two other GSK3β inhibitors (CHIR99021 and lithium chloride) to stimulate the TCF reporter gene.[12] Perplexingly, while KY02111 itself failed to nullify the ability of the two inhibitors to stimulate the reporter gene in HEK293 cells, it abrogated the reporter activation by BIO (Figure 1B). We also checked the status of β-catenin by western blot analysis. Treatment of HEK293 cells with BIO decreased the phosphorylation of β-catenin at Ser33, Ser37, and Thr41 through the inhibition of GSK3β and thereby increased the amounts of β-catenin (Figure 1C). Note that the accumulation of β-catenin is accompanied by its Ser45 phosphorylation as the CK1-mediated Ser45 phosphorylation precedes the GSK3β-mediated phosphorylation (Figure 1C). However, when the cells were co-treated with BIO and KY02111, the phosphorylation of β-catenin was maintained with a concomitant degradation of cellular β-catenin (Figure 1C). In sharp contrast, KY02111 caused no similar effects for the cells treated with CHIR99021 or lithium chloride (Figure 1C). This finding led us to conclude that KY02111 impairs the ability of BIO, but not others, to inhibit GSK3β.

With these puzzling results in hand, we next examined whether KY02111 influences the interaction of BIO with GSK3β, by taking an advantage of desthiobiotin ATP probe, which permits monitoring of ATP occupancy in kinases through its reaction with Lys residues in the ATP-binding sites[13] (Figure 2A). After treatment of cell lysate of HEK293 cells with the ATP probe, the probe-labeled proteins were purified by avidin beads and western blotted with a GSK3β antibody for estimating the ATP occupancy of GSK3β (Figure 2B). BIO, which is known to bind to the ATP-binding pocket of GSK3β, competitively displaced the ATP probe, and the competition was prevented by co-treatment with KY02111. These results and those described previously suggest that KY02111 blocks the interaction of BIO with GSK3β.

One possible explanation of the above results is that KY02111 exerts its effect by interacting directly with BIO. To test this possibility, varied concentrations of KY02111 were mixed with BIO (30 μM) in PBS containing 33% of DMSO in vitro. BIO has visible light absorbance at 507 nm exhibiting a pink color in day light, while KY02111 solutions are transparent without no apparent visible light absorption (Figure 2C–E). Addition of KY02111 displayed a decrease of the pink color and 507-nm absorption of BIO in a dose-dependent manner. Centrifugation of the samples showed precipitation of pink-colored materials at the bottom of the tubes, suggesting that KY02111 co-aggregates with BIO to form insoluble assemblies. On the other hand, SO140, a close analog of KY02111 with the amide nitrogen methylated (Figure S1), failed to co-aggregate with BIO and thereby to affect the BIO's ability to bind to GSK3β, stabilize β-catenin and stimulate the TCF reporter gene (Figures S2 and S3). When HEK293 cells were treated both with BIO and KY02111, lysed in lysis buffer, and centrifuged, similar

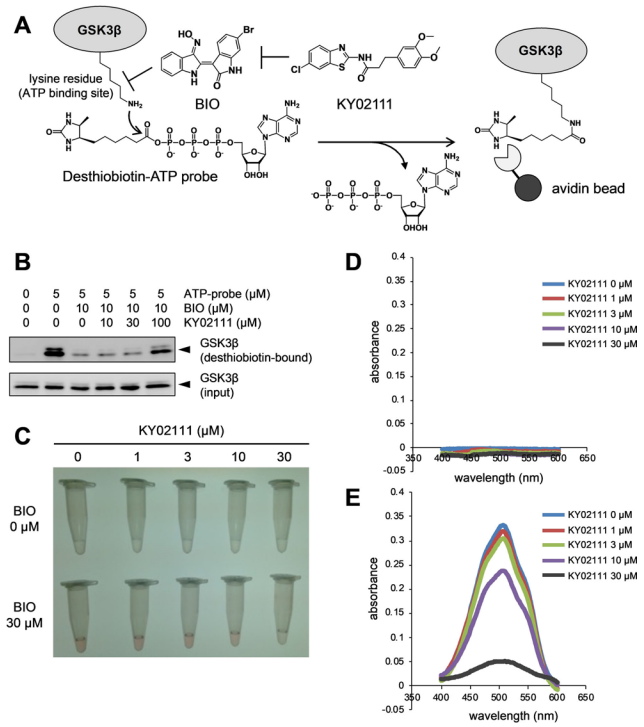

Figure 2. KY02111 forms co-aggregates with BIO. A) Schematic illustration of the desthiobiotin-ATP probe pulldown assay. B) Results of the ATP probe pulldown assay. KY02111, BIO, and ATP-probe were added to HEK293 cell lysates. After purification of destiobiotin-bound GSK3β with avidin beads, GSK3β was detected by western blotting. C–E) Co-aggregation of KY02111 with BIO. Various concentrations of KY02111 were added to a PBS:DMSO = 2:1 solution in the absence or presence of 30 μM of BIO. After mixing, photographs of the samples were captured (C), and spectra of the solutions in the absence (D) or presence (E) of BIO were measured.

insoluble pink-colored materials were observed in the pellets, supporting the notion that the two compounds co-aggregate in a cellular environment (Figure S4). These results collectively solidify the conclusion that KY02111 impairs the BIO-mediated GSK3β inhibition through co-aggregation with BIO. KY02111 does not directly inhibit endogenous Wnt signaling.

Squalene Synthase Is a KY02111-Binding Protein

Although Wnt signaling is essential during the initial stage of PSC differentiation to mesoderm cells, it needs to be repressed for increasing the yield of cardiomyocytes from mesoderm cells.[4] In the xeno-free production of cardiomyocytes from PSCs where BIO is often used as a substitute for costly Wnt ligands, initial pharmacological activation of Wnt signaling by BIO must be removed for the late stage differentiation from mesoderm cells to cardiomyocytes. Such a control over BIO can be achieved by addition of KY02111 at the late stage of differentiation. Thus, the co-aggregation of KY02111 with BIO may provide a mechanistic rationale for the observed KY02111-induced enhancement of cardiomyogenesis in a BIO-containing culture medium. However, KY02111 potentiates the generation of cardiomyocytes even in the absence of BIO, suggesting the existence of a BIO-independent mode of activation by KY02111.[3]

To obtain structure–activity profiles, we chemically synthesized eight analogs of KY02111 and examined their ability to potentiate cardiomyogeneis and to block the BIO-induced Wnt signaling (Figure S1). Removal of the Cl group or methylation of the amide group abolished the cardiomyogenesis activity, while substitution of Cl with Br or I (SO087 or KY-I, respectively) maintained or enhanced the activity. The potent activity of KY-I was lost or diminished with the modification of its dimethoxyphenyl group (SO2123, SO3027, and SO2127) or methylene linker (SO3030). On the other hand, TCF reporter assays showed that SO2068 and SO2123 readily inhibited the BIO-induced Wnt signaling although they had no detectable cardiomyogenesis activity. The structure–activity profiles were not necessarily paralleled with the BIO inhibitory activities (Figure S1). We thus hypothesized that KY02111 has a distinct endogenous target unrelated to BIO or GSK3β.

To identify a *bona fide* target of KY02111, we designed a photo-affinity probe of KY02111. Among the KY02111 analogs we synthesized, SO2093 (Figure 3A) exhibited cardiomyogenesis activity at a comparable level to that of KY02111 (data not shown), suggesting a conjugation site suited for a probe design. Conjugation of a benzophenone photoreactive group and an alkyne tag at that site yielded a photo-affinity probe, SOB1102 (Figure 3A). The benzophenone group would permit covalent photoaffinity labeling of the cellular target while the alkyne tag would facilitate identification of the photo-crosslinked proteins. HEK293 cells were treated with the probe, followed by UV irradiation and lysis of the cells. The lysates were separated into a PBS-soluble cytosolic fraction (fraction S) and an insoluble 1% NP-40-containing membrane fraction (fraction I). After Cu-catalyzed azide-alkyne cycloaddition with a rhodamine-azide molecule, probe-crosslinked proteins were separated by SDS-PAGE and visualized by in-gel fluorescence scanning (Figure 3B). We found a ≈45 KDa band in the insoluble membrane fraction that was competed out with an excess amount of KY02111 (indicated by an arrow). The ≈45 KDa protein was purified by avidin agarose resins after click reaction with a biotin-azide. Mass spectrometric sequencing identified the 45 KDa protein as squalene synthase (SQS), an ER-membrane enzyme that catalyzes the first step committed to the biosynthesis of sterols within the isoprenoid pathway. Western blot analysis confirmed the binding of probe to SQS, and the interaction was competed with an excess amount of KY02111 (Figure 3C). Importantly, the interaction between SQS and the probe was also prevented by the addition of KY-I, a KY02111 analog that exerts cardiomyogenesis more potently than KY02111, but no effects was observed with SO3030, which is devoid of cardiomyogenesis activity (Figure 3D).

Cellular engagement of KY02111 with SQS was validated by cellular thermal-shift assays, in which thermal stabilization of proteins upon ligand binding is monitored in a cellular context. SQS protein was denatured upon heating the cells at 55 °C and thereby precipitated out from the soluble fraction.

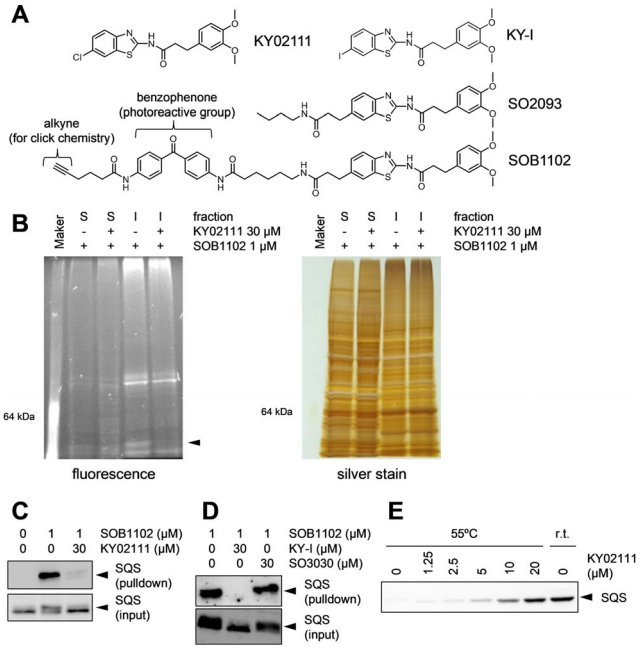

Figure 3. Identification of squalene synthase as a binding protein of KY02111. A) Structures of KY02111, KY-I, SO2093, and SOB1102. B) Photoaffinity labeling. HEK293 cells were pretreated with 0 or 30 μM of KY02111 for 20 min and treated with 1 μM of SOB1102 for 40 min. After UV irradiation for 30 min, the cells were collected and fractionated into PBS-soluble (S) and PBS-insoluble (I) fractions. To each fraction, rhodamine-azide was added for click chemistry. The rhodamine-conjugated proteins were separated by SDS-PAGE and visualized by in-gel fluorescence scanning (left). Whole proteins were detected by silver stain (right). C, D) Confirmation of photo-reacted SQS. HEK293 cells were pretreated with DMSO, KY02111 (30 μM) (C), KY-I (30 μM) (D), or SO3030 (30 μM) (D) for 20 min and treated with 1 μM of SOB1102 for 40 min. After UV irradiation for 30 min, the cells were collected. To the PBS-insoluble fraction, biotin-azide was added for click chemistry. After purification of biotin-conjugated proteins with avidin beads, SQS was detected by western blotting. E) Cellular thermal-shift assay. HEK293 cells were treated with various concentrations of KY02111 for 1 h. The cells were collected and treated with heat at 55 °C. The cell extracts were immunoblotted with an α-SQS antibody.

When the cells were treated with increasing amounts of KY02111, more SQS protein remained in the soluble fraction (Figure 3E), corroborating that KY02111 binds to SQS in cells. The eight analogs of KY02111 were also subjected to cellular thermal-shift assays. Analogs SO087 and KY-I, which exerted cardiomyogenesis as much as or greater than KY02111, respectively, stabilized SQS at similar or higher levels than that of KY02111 (Figures S1 and S5). On the other hand, the analogs that had little cardiomyogenesis activity failed to thermally stabilize SQS in cells. The ability of the bioactive analogs to potentiate cardiomyogenesis mirrored their levels of thermal stabilization of SQS in cells (Figure S5B). The excellent correlation encouraged us to further pursue SQS as the most likely target protein of KY02111.

KY02111 Binds to SQS without Affecting Its Enzymatic Activity

SQS is a mevalonate pathway enzyme that catalyzes the conversion of farnesyl diphosphate to squalene for the biosynthesis of sterols. We first examined whether KY02111 has affinity to SQS(31–370), a truncation mutant of SQS encompassing the enzyme domain (Figure S6A). The photoaffinity probe of KY02111 (SOB1102) exhibited photoreaction with FLAG-tagged SQS(31–370) as much as it did with FLAG-tagged full-length SQS in HEK293 cells (Figure S6B), and the reaction was competed with an excess amount of KY02111 (Figure S6C). To confirm the direct interaction between SQS(31–370) and KY02111, we prepared SOB1046, a fluorescein-conjugated fluorescent probe of KY02111, and examined its association with bacterially expressed SQS(31–370) in vitro. Addition of SQS(31–370) dose-dependently increased the degree of fluorescent polarization of SOB1046, indicating the reduced rotation of SOB1046 due to its physical association with the SQS protein. The K_D value was estimated to be 167.2 nM (Figure S7).

The direct interaction of KY02111 with SQS(31–370) prompted us to examine the effects of KY02111 on the enzymatic activity of SQS in vitro. The SQS enzymatic activity was monitored by measuring the production of $NADP^+$, which is accompanied with the conversion of farnesyl diphosphate to squalene (Figure S8). Unexpectedly, KY02111 failed to display detectable inhibitory effects on the production of $NADP^+$ up to 30 μM, whereas zaragozic acid (ZA-A), a natural product that is known to inhibit the enzymatic activity of SQS,[14] readily impaired the production of $NADP^+$ in a dose-dependent manner (Figure S8B). The effects of KY02111 on the SQS activity was further evaluated in cells by monitoring the transcriptional activation capability of sterol regulatory element-binding protein (SREBP) (Figure S8C). Lipid-depletion from the medium or addition of compactin (an HMG-CoA reductase inhibitor) or ZA-A each increased the signals from an SREBP reporter gene by limiting the intracellular levels of sterols, endogenous inhibitors of SREBP. In contrast, KY02111 had no detectable effects on the SREBP activity up to 30 μM (Figure S8C). These results indicate that KY02111 directly binds to SQS without affecting the SQS enzymatic activity in vitro and in cells.

KY02111 Impairs TGFβ Signaling by Disrupting the SQS-TMEM43 Interaction

The inability of KY02111 to modulate the enzymatic activity of SQS prompted us to examine its effects on the protein-protein interactions of SQS. The protein-protein interaction database of BioGRID indicates four potential SQS-interacting proteins that are localized in ER: SRPRB,[15] ARL6IP1,[16] SYVN1,[17] and TMEM43.[18] The Myc-tagged versions of these proteins were expressed in HEK293 cells, and their potential interactions with FLAG-tagged SQS were evaluated by co-immunoprecipitation (Figures 4A and S9). The results showed that SQS associates with TMEM43 but not with SRPRB, ARL6IP1, and SYVN1. The SQS-TMEM43

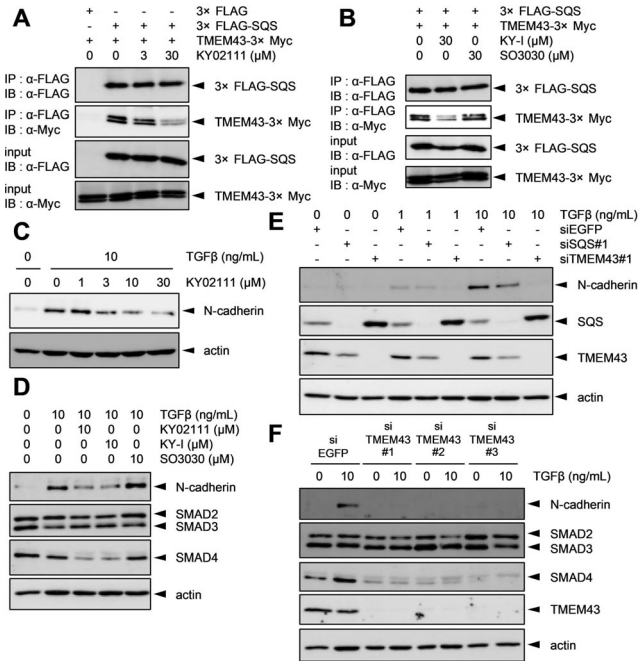

Figure 4. KY02111 affects TGFβ signaling by disruption of SQS-TMEM43 interaction. A, B) Effects of KY02111 and its derivatives on the interaction between SQS and TMEM43. HEK293 cells were transfected with pCMV-3Tag-1a or pCMV-3Tag-1a-SQS, and pCMV-3Tag-9-TMEM43. After 6 h, various concentrations of KY02111 (A), DMSO, KY-I (30 μM), or SO3030 (30 μM) (B) were added. Immunoprecipitated proteins were analyzed by immunoblotting. C, D) Effects of KY02111 and its derivatives on the TGFβ-induced expression of N-cadherin. A549 cells were treated with TGFβ (0 or 10 ng mL^{-1}) for 2 days and then with various concentrations of KY02111, DMSO, KY02111 (10 μM), KY-I (10 μM), or SO3030 (10 μM) for another 2 days. The lysates were analyzed by immunoblotting. E) Effects of SQS/TMEM43 knockdown on the TGFβ-induced expression of N-cadherin. A549 cells were transfected with each siRNA (siEGFP, siSQS, or siTMEM43). After 2 days, the cells were then treated with various concentrations of TGFβ for another 2 days. The lysates were analyzed by immunoblotting. F) Effects of TMEM43 knockdown on the SMADs expression. A549 cells were transfected with each siRNA (siEGFP or siTMEM43). After 2 days, the cells were treated with 0 or 10 ng mL^{-1} of TGFβ for another 2 days. The lysates were analyzed by immunoblotting.

interaction was abolished by addition of KY02111 or KY-I but not SO3030, a KY02111 analog that lacks in cardiomyogenesis activity, suggesting that the pharmacological disruption of the SQS-TMEM43 interaction is related to the cardiomyogenesis activity (Figures 4A and 4B).

During the course of our analysis, the biological activity of KY02111 was also profiled by a series of cellular assays in the MEXT molecular profiling platform program, a nation-wide consortium for profiling biological activities of small molecules. KY02111 was found to dose-dependently diminish the TGFβ-induced expression of N-cadherin in human LoVo colon adenocarcinoma cells. Similar results were reproduced in human A549 lung cancer cells (Figure 4C), and KY-I, but not SO3030, also decreased the TGFβ-induced expression of N-cadherin (Figure 4D).

The functional link between TMEM43 and TGFβ has not been well-documented in the literature. To probe this possibility, we examined the effects of siRNA knockdown of TMEM43 or SQS on the TGFβ-induced expression of N-cadherin (Figure 4E). The knockdown of either TMEM43 or SQS suppressed the TGFβ-induced expression of N-cadherin, although the suppression was weaker in the SQS knockdown. Interestingly, the SQS knockdown lowered the protein level of TMEM43. It is likely that the physical association of TMEM43 with SQS stabilizes TMEM43 to upregulate the TGFβ signaling.

TGFβ cytokines regulate gene expression by receptor-mediated activation of SMAD transcription factors. Receptor binding of a TGFβ cytokine stimulates the phosphorylation of SMAD2 and SMAD3 to form a hetero-trimeric complex with SMAD4 that binds to DNA with partner transcription factors. Three independent siRNA knockdown of TMEM43 in A549 cells all reduced the protein level of SMAD4 while a control siRNA of EGFP had no significant effects (Figure 4F). The protein levels of SMAD2 and SMAD3 were unchanged, indicating that the TMEM43 knockdown selectively eliminates SMAD4. Treatment of the cells with KY02111 or KY-I similarly led to the decreased protein level of SMAD4 but not SMAD2 and SMAD3, while SO3030, an inactive analog of KY02111, showed no detectable effects on the protein levels of SMADs (Figure 4D). Overall, our data led us to propose a model in which KY02111 decreases the protein level of SMAD4 and thereby represses TGFβ signaling by destabilizing TMEM43 through the disruption of the SQS-TMEM43 interaction. Mounting evidence indicates that TGFβ signaling plays a key role in cardiomyogenesis, and inhibitors of TGFβ receptors have been reported to promote cardiomyogenesis.[19] KY02111 potentiates cardiomyogenesis most likely by inhibiting TGFβ signaling.

Prolonged KY02111 Exposure Induces ARVC-like Phenotype in Cells

TMEM43 is a nuclear and ER membrane protein that is conserved from bacteria to human.[18] Although the biological roles of TMEM43 remain unclear, its mutations lead to arrhythmogenic right ventricular cardiomyopathy (ARVC), an inherited heart disease that involves a substitution of myocardium with fatty tissue. The most aggressive arrhythmogenic cardiomyopathy/ARVC subtype is ARVC type 5 (ARVC5), caused by a S358L mutation in TMEM43.[20] Mice expressing TMEM43-S358L recapitulate the human disease, displaying cardiomyocyte death with severe fibrofatty replacement.[21] These clinical and experimental observations of TMEM43 properties led us to investigate the effects of the S358L mutation on the interaction with SQS (Figure S10). Co-immunoprecipitation experiments showed that the mutated TMEM43 failed to associate with SQS, suggesting that the disrupted interaction of TMEM43 with SQS might be related to the ARVC phenotype.

We speculated that the continued disruption of the SQS-TMEM43 interaction by KY02111 during the entire process of cardiomyogenesis may also lead to the ARVC phenotype. To test the hypothesis, we added KY02111 to the culture medium throughout an 18-day cardiomyogenesis protocol of human iPS cells (Figure S11A). As previously reported, this

established protocol generated 80% cTNT positive cells with high *TNNT2* expression levels. The expression levels of these two cardiomyocyte markers were significantly reduced by the consecutive treatment with KY02111 (Figure S11BC). The KY02111-treated cells displayed increased levels of preadipocyte markers (*DLK1* and *PPARG*) (Figure S11DE). SO3030, a KY02111 analog that does not affect the SQS-TMEM43 interaction, had limited effects. Taken together, these data suggest that the disruption of the SQS-TMEM43 interaction contributes to the ARVC phenotype and that this KY02111-induced activity serves as a tool to mimic the ensuing disease.

Discussion

Our chemical genetic study demonstrates that KY02111 and its optimized analog KY-I bind to SQS and thereby impair TGFβ signaling through the disruption of the SQS-TMEM43 interaction. This mechanism is fully consistent with the ability of KY02111 to boost mesodermal-to-cardiomyocyte differentiation. The timing of TGFβ signaling blockade has been shown to be important for inducing cardiomyogenesis.[19a] Adding an inhibitor of TGFβ signaling during the differentiation of mesodermal cells into cardiomyocytes promotes myocardial differentiation. In contrast, the addition of a TGFβ signaling inhibitor during iPS cell differentiation into mesodermal cells does not display such pro-differentiation activity. Importantly, our study showed that KY02111 exposure throughout cardiomyogenesis induces ARVC-like phenotype in cells, analogous to the phenotype of clinically observed TMEM43 mutation. The findings suggest the interesting possibility that the prolonged disruption of the SQS-TMEM43 interaction contributes to the ARVC phenotype and that KY02111 serves as a unique tool to mimic the disease.

In addition to the mutations of TMEM43, those of several other genes have clinically been identified as responsible for the development of ARVC. Among them are TGFβ3,[22] a member of the TGFβ superfamily, and desmosome constituents such as desmoplakin,[23] plakophilin-2,[24] desmoglein-2,[25] desmocollin-2[26] and plakoglobin,[27] which are important for cell-cell adhesion and are abundant in the epidermis and myocardium. The expression of plakoglobin, desmoplakin, and plakophilin-2 is regulated by TGFβ signaling.[28] Thus, myocardial degeneration and fibrosis in patients with ARVC may be the result of dysfunction and disruption of intercellular adhesion structures due to abnormal TGFβ signaling. The present study revealed that TMEM43 also plays a key role in TGFβ signaling through its interaction with SQS. Significantly, the SQS-TMEM43 interaction is disrupted by the clinically observed mutation of TMEM43. The TMEM43 mutation contributes to the development of ARVC most likely through dysregulation of TGFβ signaling.

Our results underscore an unexpected role of SQS in TGFβ signaling. SQS is the traditional metabolic enzyme that has long been known to convert FPP to squalene for cholesterol biosynthesis. SQS knockout mice are embryonic lethal, indicating that SQS is essential for development.[29] However, even when pregnant mice carrying SQS knockout mice are given excessive amounts of squalene and cholesterol in their diet, SQS knockout mice remain embryonic lethal,[29] suggesting that SQS has other important developmental functions besides the synthesis of squalene. The regulation of TGFβ signaling through its interaction with TMEM43 may represent one of those additional functions.

Another unexpected lesson arising from our study is due to the surprising finding that KY02111 exerts its effect by inhibiting the activity of BIO through molecular co-assembly. BIO has a structure with two linked indole rings. Such a chemical feature can be found in indigo, which in fact similarly co-assembled with KY02111 in vitro (data not shown). In contrast, tryptophan, which has only one indole ring, failed to aggregate with KY02111 (data not shown). Presumably, KY02111 aggregates and squelches small molecules bearing two indole rings. Small-molecule targeting by a bioactive small-molecule has been barely documented, except by those of theonellamide[30] and adhesamine,[31] which exert their biological activity by binding to ergosterol and heparansulfate, respectively. The KY02111-BIO interaction presents an example in which two exogenous small molecules selectively interact and condensate each other. Such finding would complicate the understanding of pharmacological and chemical biological investigations, as exemplified in our study. Our limited experience serves as cautionary warnings of the risks in using multiple small molecules simultaneously in cellular and molecular investigations.

Our work has generated several important questions that will be the basis for further deep investigations. Foremost, we do not understand how TMEM43 maintains the SMAD4 protein levels. In canonical TGFβ signaling, SMAD4 serves as a central mediator in transmitting the signal to the nucleus. Its expression level is tightly regulated and frequently decreased in human cancer. Ubiquitin (E3) ligase Skp2[32] and β-TrCP1[33] interacts with SMAD4, which leads to the increased ubiquitination and accelerated proteolysis. It has also been reported that BRK (BRK-Y447F) phosphorylates SMAD4,[34] resulting in its degradation through the recognition by the ubiquitin-proteasome system. We found that knockdown of TMEM43 decreases the protein levels of SMAD4 (Figure 4D), suggesting that TMEM43 activates TGFβ signaling by maintaining the steady-state SMAD4 level. Future work will be critical for a better understanding of the underlying molecular basis for the finding.

Another question is how SQS stabilizes TMEM43 through protein-protein interaction. Protein-protein interactions of ER-membrane bound proteins often increase protein stability. A representative example is the physical association of SREBPs with SREBP cleavage-activating protein (SCAP), an escort protein of SREBPs, which stabilizes SREBPs by masking their degradation signals.[35] Another interesting case is HMG-CoA reductase (HMGCR), an ER-membrane-bound rate-limiting enzyme in mevalonate pathway. Under low geranylgeranyl pyrophosphate (GGPP) concentrations, ER-resident UbiA prenyltransferase domain-containing protein 1 (UBIAD1) binds and stabilizes HMGCR. Under GGPP excess, UBIAD1 translocates to the Golgi, releasing HMGCR for proteasomal degradation.[36] Our future studies

will focus on elucidating the molecular underpinning of the TMEM43 stabilization by SQS.

Furthermore, we need more fully to understand how KY02111 displayed weak downregulation of Wnt-dependent genes in our earlier transcriptome analysis,[3] which was the reason for focusing our initial studies on Wnt signaling as a potential target pathway of KY02111. Efficient differentiation of mesoderm cells into cardiomyocytes requires the inactivation of BMP, activin, and TGFβ signaling as well as Wnt signaling.[19b] Ample studies have described the signaling crosstalk among these signal transduction pathways. BMP, Activin, TGFβ signalings share a number of SMAD members including SMAD4 in transmitting the signals.[37] The Wnt-signaling scaffolding protein Axin and its associated kinase, GSK3β, interact with TGFβ-regulated SMAD3 for proteasome-dependent degradation. One of the downstream transcription factors in Wnt signaling, lymphoid enhancer factor 1 (LEF1), is activated by phosphorylated SMAD2 and SMAD4 to control the E-cadherin gene expression,[38] while the protein stability of Snail, a downstream factor of TGFβ signaling, is regulated by GSK3β through phosphorylation.[39] These previously reported crosstalk between Wnt and TGFβ signaling pathways and the findings of the present study indicate that KY02111 indirectly affects Wnt signaling through the decrease of SMAD4 levels.

Conclusion

Our mechanistic study of KY02111 and its analogs revealed a heretofore undescribed critical role of SQS in TGFβ signaling. It shows that KY02111 disrupts the SQS-TMEM43 by binding to SQS and thereby recapitulates an ARVC familial mutation of TMEM43. KY02111 and its analogs could be used in ARVC modeling, as well as serve as a research tool for studying TGFβ/SMAD signaling. The findings of the present study provide a fresh perspective for the study of the TGFβ signaling pathways and serve as a starting point for further investigations in more physiological settings.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: cardiomyogenesis · chemical genetics · SQS · TGFβ signaling

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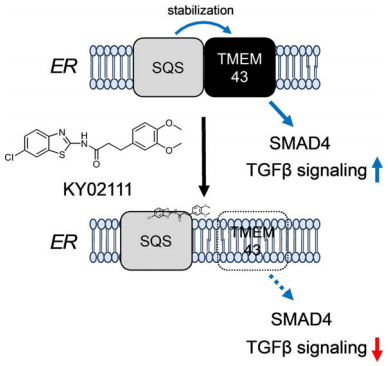
Research Articles

Cell Signaling

Y. Takemoto, S. Kadota, I. Minami, S. Otsuka, S. Okuda, M. Abo, L. L. Punzalan, Y. Shen, Y. Shiba,* M. Uesugi* ——— ▮▮▮▮–▮▮▮▮

Chemical Genetics Reveals a Role of Squalene Synthase in TGFβ Signaling and Cardiomyogenesis



KY02111 is a widely used small molecule that boosts cardiomyogenesis. Chemical genetics of KY02111 identified squalene synthase (SQS) as a molecular target of KY02111. By disrupting the interaction of SQS with cardiac ER-membrane protein TMEM43, KY02111 impairs TGFβ/SMAD signaling and recapitulates the clinical mutation of TMEM43 that causes an inherited heart disease.